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Aflatoxins in Almonds

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ABSTRACT

Aflatoxins are associated with almond kernels damaged by the navel orangeworm (NOW) larva(e). The NOW attacks the almond fruit after hull-split while they are drying on the tree. During drying, high temperatures in the orchard and moisture in the hulls provide an environment especially suited for the growth of some fungi. These conditions, in combination with injury caused by the NOW, favor the growth of *Aspergillus flavus* Link and *A. parasiticus* Speare, fungi that may produce carcinogenic aflatoxins. Aflatoxin production is influenced by (1) the kind of fungi present on the hull, (2) temperature, (3) available moisture, and (4) the maturity of the hull or kernel on which the fungi grow. The reduction of insect damage after hull-split and during the drying of the fruit would be the most practical means of minimizing the aflatoxin content of almonds in the orchard.

KEYWORDS: Aflatoxin, almonds, *Aspergillus flavus*, *Aspergillus parasiticus*, carcinogens, navel orangeworm.

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AFLATOXINS IN ALMONDS

By Douglas J. Phillips, Steven L. Purcell, and George I. Stanley[1]

INTRODUCTION

Aflatoxins are a specific group of chemicals produced by two fungi, *Aspergillus flavus* Link and *A. parasiticus* Speare (AF) (*35*).[2] At least 18 chemicals are known as aflatoxins. The more common aflatoxins are named aflatoxin B-1, aflatoxin B-2, G-1, and G-2. They are mutagenic, carcinogenic, teratogenic, and acutely toxic to most animals and humans (*36*). The exact amount of aflatoxin that is dangerous to humans is not known; however, 20 parts per billion (p/b) is the maximum permissible guideline level for aflatoxins in food commodities sold in the United States (*41*).

The aflatoxins found in almonds (*41*) are usually associated with damaged kernels (*19*, *31*, *37*). When the damaged kernels are removed from a sample of almonds, virtually all the aflatoxins are removed with them (*19*). A large proportion of damage to almond kernels is caused by *Amyelois transitella* (Walker), the navel orangeworm (NOW). This damage greatly increases the incidence of AF in the kernels. The NOW attacks almonds in the orchard at or near the time of hull-split (*14*). Hull-split is the natural splitting of the fruit as it matures on the tree (*47*). At hull-split, there is ample moisture in the hull and the kernel to support fungal growth. Generally, kernels that contain above 7-percent moisture will support fungal growth. At hull-split, we found the moisture content of the kernels to be as high as 48 percent of their fresh weight. The spores of the *Aspergilli* can be easily found on the hull and are carried there by the wind or by other natural means of dissemination (*34*). These spores on the surface could be picked up and carried by the NOW into the kernel.

TESTING NAVEL ORANGEWORM FOR *ASPERGILLUS FLAVUS*

In order to determine if the NOW carries spores inside or on the surface of its body, we collected samples of larvae that were foraging on almond kernels.

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[2] Italic numbers in parentheses refer to Literature Cited, p. 8.

In laboratory studies, five collections of worms were made from AF inoculated or noninoculated kernels. Laboratory insects were reared from surface sterilized eggs, and the NOW larvae foraged several weeks on the kernels before the larvae were tested for fungi. In field studies (10 collections), we used larvae collected from almonds on the tree or from almonds that had been harvested and were awaiting hulling. For each sample, whether from orchard or laboratory, we collected 50 NOW larvae and left them in a clean beaker overnight. The frass found in the beaker the next morning was suspended in cold, 0.1-percent water-agar (liquid) and plated onto Bell and Crawford's *Aspergillus flavus* isolation medium with 50 mg/L DCNA (2,6-dichloro-4-nitroaniline) added (*8*).

The larvae were washed for 15 min in cold, 0.1-percent water-agar, and an aliquot of the water-agar was plated. The larvae were surface desinfested with 0.5-percent sodium hypochlorite for 5 min, and the hypochlorite was drained from them. They were then homoginated in 0.1-percent water-agar, and the homoginate was plated. The inoculated plates were incubated 7 days at 30°C, and the AF colonies were counted.

Some of the NOW larvae grown on kernels in the laboratory had AF on their surface, in the frass, and within their bodies. These AF were found on kernels where AF was abundant (table 1). Neither the 500 NOW larvae collected in the field nor their frass yielded any AF. Thus, while the AF spores may be carried by the NOW larvae, they rarely or seldom carry the fungus under field conditions. The NOW larvae, although associated with the aflatoxin problem in the orchard, appear at most to be only an occasional or nonspecific vector of AF.

Table 1.--*The number of* Aspergillus flavus *or* A. parasiticus *colonies found on the surface, in the frass, or in the homogenate of navel orangeworm larvae from infested almonds*

Type of sample and date in 1975	Number of colonies per 50 larvae[1]		
	Frass	Surface	Homogenate
Noninoculated:			
April 23	0	0	0
May 23	1,000	100	100
Inoculated:[2]			
June 4	3,000	100	1,500
June 13	900,000	18,000	30,000
June 20	14,000	6,000	5,000

[1]The almonds were infested in the laboratory with eggs from adult navel orangeworm.

[2]Inoculated with dry conidia of *A. flavus* and *A. parasiticus* before insect infestation.

Apparently, one role of the NOW larvae is to injure the almond fruit, thereby providing an opening into the kernel where AF spores may be carried or disseminated by several factors in the environment such as wind, rain, and other insects. The NOW-AF relationship on almonds appears to be similar to that of the pink bollworm and AF on cotton (*1*, *3*, *4*, *5*).

INVASION OF THE KERNEL

Aspergillus flavus and *A. parasiticus* are primarily saprophytes, requiring dead tissue to grow upon (*1*, *15*). These fungi also require less moisture than most fungi (*12*, *13*). As the almond fruit dries and tissues die (due to insect damage or natural senescence), conditions favor toxin production by AF in the tissue. When almond hulls were inoculated with AF at or after hull-split, in the presence or absence of the NOW larvae, AF penetrated into the kernels and produced aflatoxin (*31*); however, at harvest, the almond was too dry for AF activity because of the moisture content of the kernels had dropped below 5 percent (*31*). This relationship suggests that aflatoxin was produced in the orchard after hull-split but before harvest.

The activity of NOW may continue after harvest, and this activity may provide moisture that would prolong fungal growth and toxin production. To evaluate toxin production after harvest of the almonds, but before their storage, an orchard near Bakersfield, Calif., was selected that had a history of NOW damage. In this area, AF was commonly found on sound hulls and kernels (*34*) as well as on damaged ones. In this orchard, we fumigated harvested almonds with 0.02 percent of hydrogen phosphide per cubic meter for 24 hr to stop NOW activity. The harvested almonds were fumigated: (1) about a week before they would normally have been harvested, (2) immediately after normal harvest, and (3) several weeks after harvest and just prior to removing the hull. This test included six plots each containing the three fumigation treatments. After thorough drying, the hulls and shells were removed from the harvested almonds. Each plot yielded about 15 to 25 lb of kernels per replication. The damaged kernels, which comprised 1 to 2 percent of the sample, were sorted out, ground, and analyzed for aflatoxin (*40*).

Seven of the 18 worm-damaged samples contained aflatoxins at concentrations ranging from 2.9 to 1,346 p/b (table 2). The aflatoxin did not occur at significantly different levels in kernels from the three harvest-fumigation treatments. Because the aflatoxin content was similar in the three treatments and because earlier studies (*14*, *31*) indicated that neither AF nor NOW larvae invade almonds before hull-split, we conclude that aflatoxin production in the orchard generally occurs after hull-split, but before the kernels are harvested from the tree; this preharvest period is critical for aflatoxin production.

THE INFLUENCE OF ENVIRONMENT

Competitive Fungi

Many micro-organisms may be found on almonds (*25*, *26*, *29*, *44*). A 1971 survey of almond orchards throughout the Central Valley of California suggested

Table 2.--*The aflatoxin content of worm-damaged 'Nonpareil' almonds fumigated on 3 dates in the orchard*

Harvest date and fumigation group	Kernels with worm damage[1]	Number of wormy kernels with *Aspergillus flavus* visible	Aflatoxin content of worm-damaged kernels
	Percent	*Moldy kernels per total kernels*	*Parts per billion*[2]
One week before normal harvest, Aug. 9[3]	1.3	0/150	n.d.
	1.0	0/125	51
	1.2	0/125	n.d.
	1.1	0/150	n.d.
	1.2	1/150	n.d.
	1.3	1/200	254
Total		2/900	
Normal harvest, Aug. 21[4]	2.5	0/300	3
	2.1	2/250	199
	1.4	1/200	64
	1.6	0/175	n.d.
	1.2	1/150	n.d.
	1.1	0/150	n.d.
Total		4/1225	
Two weeks after normal harvest, before hulling, Sept. 1	1.1	0/100	n.d.
	1.1	0/150	n.d.
	2.2	1/200	n.d.
	2.4	1/200	83
	.9	1/100	1346
	.9	1/ 75	n.d.
Total		4/825	

[1] From 15- to 25-lb samples.
[2] n.d. = less than 2 p/b.
[3] Also fumigated on Aug. 21 and Sept. 1
[4] Also fumigated on Sept. 1.

that AF is influenced by the location of the orchard and by the presence of other fungi that also occur on the hull and kernel (*32*, *34*). Fungi that may interact with AF have been reported on various media or crops (*6*, *9*, *10*, *22*, *23*, *38*, *45*). In orchard experiments, almond fruits were inoculated with AF alone or in combination with other fungi that were potentially antagonistic to AF. We found that the presence of certain other fungi on the almond hull reduced the number of isolations of AF that were recovered from hulls or kernels. Fungi that require high moisture appeared to be most antagonistic to AF when colonizing almonds kernels. The fungus *Ulocladium chartarum* (Pr.) Simmons reduced the isolation of AF most significantly (*32*). While this study showed antagonists are able to reduce the colonization of AF, it did not show that a competitive organism could reduce toxin production by AF on almonds in the orchard. In a separate test, the fruit of almond cultivar 'Nonpareil' were inoculated at the beginning of hull-split with dry conidia of aflatoxin producing *Aspergilli* alone or in combination with *U. chartarum* using the previously described method (*32*). Almond fruit on 45 trees were inoculated with these fungi and then enclosed in a filter bag, 10 to 12 fruit per bag. The treatments included AF alone, *U. chartarum* alone, the combination of AF and *U. chartarum*, and an uninoculated control. All fruit remained on the tree until normal harvest. At harvest, the bags containing the fungi and the almonds were cut from the tree unopened. Five samples of 90 hulls or kernels from each treatment were ground and analyzed for aflatoxin using minicolumn detection methods (*33*, *40*).

The presence of *U. chartarum* reduced the average amount of aflatoxin in the hull from 161 to 61 p/b and in the kernel from 2 p/b to an undetectably low level (table 3). These results (1) indicate that the reduction in the colonization of the hull and kernel by AF was accompanied by reduced toxin production and (2) emphasize the importance of competitive organisms in the natural control of aflatoxin in the field.

An earlier report showed that a significant amount of aflatoxin occurred in samples to which no NOW were added (*31*); however, these results did not necessarily reflect aflatoxin preduction in sound kernels, because the technique used did not totally eliminate natural NOW infestation. In our tests to evaluate the effects of competitive fungi, sound almond kernels inoculated only with AF contained an average of only 2 p/b aflatoxin (table 3). This result supports observations (*19*, *37*) that sound almond kernels are relatively safe and toxin free as they come from the orchard.

Temperature and Moisture

High temperatures tend to favor the occurrence of AF on almonds (*34*) and on other crops (*2*, *20*, *24*, *39*). The AF are able to grow at relatively high temperatures and reduced moisture levels when compared with other fungi (*21*). The lowest temperature reported for growth of AF is 6°C, the highest temperature supporting growth is to 46°, and the temperature where optimum growth occurs is between 36° and 38° (*42*). These cardinal temperatures, determined at or near 100-percent relative humidity (RH), depend somewhat on the moisture activity. For example, at 78-percent RH, the highest temperature supporting growth was 43° (*7*). Spores produced by AF fungus also may be inactivated or killed by high temperature (*18*). Inactivation under moist heat at 50° to 55° occurs in 3 min,

Table 3.--*Aflatoxin in undamaged almond hulls and kernels. The hulls were inoculated before hull-split with dry spores or mycelium of 4 toxicogenic isolates of* Aspergillus flavus *or* A. parasiticus *in the presence or absence of* Ulocladium chartarum. *The samples were analyzed for aflatoxin after 2 months of dry storage*

Fruit part and replicate	*A. flavus* and *A. parasiticus*	*A. flavus* *A. parasiticus* and *U. chartarum*	*U. chartarum*	No fungus added
	Total aflatoxins (ng/g)[1]			
Hulls:[2]				
1	272.0	146.5	n.d.[3]	n.d.
2	294.5	34.0	--do--	Do.
3	17.5	17.5	--do--	Do.
4	145.0	.8	--do--	Do.
5	76.5	107.0	--do--	Do.
Mean	161.1	61.2	--do--	Do.
Kernels:				
1	6/6	n.d.	n.d.	n.d.
2	n.d.	--do--	--do--	Do.
3	--do--	--do--	--do--	Do.
4	2.8	--do--	--do--	Do.
5	n.d.	--do--	--do--	Do.
Mean	1.9	--do--	--do--	Do.

[1] 1 ng = 1 billionth of a gram.
[2] Each sample contained 90 kernels or hulls.
[3] n.d. = less than 2 p/b.

whereas, under dry conditions, it may take nearly 1,000 min (*17*). The optimum moisture condition for spore germination also may change with temperature changes (*11*).

These complex moisture and temperature relationships influence the occurrence of AF and of other fungi on almonds in the orchard. In California orchards, the temperature at various sites in orchards is often above 45°C, a level that appears to favor occurrence and growth of AF (see appendix table 1). AF is found most frequently in the warmer, southern regions of the Central Valley of California (*34*, *43*). In contrast, some fungi, for example, *Ulocladium chartarum*, occur less frequently in these warmer areas (*34*). Even within a given orchard, more AF was found in warm, sunny sites than in cooler, shaded

sites (*34*). These effects of temperature are difficult to separate from the related effects of moisture. The AF begin growth on the hull at hull-split when some tissues age and die or are injured by insect attack, and they may continue growing until the available moisture becomes a limiting factor. A drop in water activity to 0.82 or 82-percent RH is usually considered sufficient to stop growth of AF (*30*). This level probably occurs at or near the time the almonds are harvested (*31*, *46*). As the almonds dry below a water activity of 0.82, the laboratory isolation of AF from almonds increases (*34*). This increase may be in part an artifact of the isolation technique, which is influenced by the presence or absence of other fungi.

The Interaction of Competitive Fungi, Temperature, and Moisture

The amount of domination of groups of fungi over the fungal population on almonds depends on the ability of the fungi to colonize, grow, and survive on the drying fruit (*34*, *44*). At the beginning of the drying period, moisture requiring fungi may dominate; however, as the drying continues, fewer and fewer of these moisture requiring fungi survive, and, by the time the almonds reach the storage bin after an uninterrupted drying period, a population of relatively xerophytic storage fungi including AF, *Penicillium* sp., and other *Aspergilli* usually dominate (*34*). This progression of fungi is similar on a wide variety of crops (*12*, *13*, *16*, *18*). If the drying is interrupted by rain or another source of moisture, the rewetting stimulates the fungi that are present into competition for the remoistened substrate. The high moisture provided by rewetting may favor hydrophilic (moisture loving) fungi, or xerophilic (dry loving) fungi, depending on the relative abundance of propagules.

This effect of population density has been reported (*28*, *42*) and can be demonstrated easily with almonds by thoroughly wetting dry almonds hulls and inoculating them with a large amount of yeast. The yeast dominates and few *Aspergilli* are observed even though *Aspergilli* are normally present in large numbers on the dry hulls; however, if water is added without the yeast, *Aspergilli* will dominate and AF can be observed. This does not mean that no aflatoxin will be produced when AF and yeast are mixed and growing together, but it does show that in a mixture one can expect reduced growth of AF and reduced toxin production as compared with AF growing alone or in a dominant role.

CONTROL OF AFLATOXIN

To reduce or control the production of aflatoxin under natural conditions, one must consider the kinds and amounts of various fungi present, the temperature, the moisture, and the type of growth medium on which the fungi are growing because all together these factors influence how much and when the toxin is produced. Insect damage may cause an increase in the aflatoxin content by opening and injuring the tissue at a time critical for AF growth. The insect also may alter the substrate in a way that favors AF. The alteration most likely occurs while the almonds are still on the tree. Insect damage is by far the most important factor leading to aflatoxin production in almonds in the field. Thus, from a practical point of view, the most direct means of reducing

aflatoxin content of almonds is to reduce insect damage and to remove worm-damaged nuts by sorting after harvest (*19*, *37*). Maintaining cool, dry storage conditions (*12*, *15*) helps to maintain the quality of the sound kernels.

Cultural practices in the field also may modify the amount of aflatoxin in the almond, but further research is needed before recommendations can be made. Irrigation methods or shading of the drying nuts would tend to modify moisture and temperature in the orchard and thus, would have an impact on the kinds of fungi that occur. These cultural factors also may influence the kinds of fungi that colonize insect-damaged nuts and, consequently, reduce or increase aflatoxin production.

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APPENDIX

In 1977, various temperatures at 24 sites were monitored within two trees in an orchard near Bakersfield, Calif. Temperatures were recorded on 27 days for 12 h a day, from 8 a.m. to 8 p.m. The temperatures of the almonds were measured while they were on the tree for 18 days, from August 8 to 26, and while they were on the ground under the trees for 9 days, from August 27 to September 4. Temperatures of almonds were measured with a thermocouple placed between the almond shell and kernel, midway between the dorsal and ventral edge of the almond fruit. The thermocouples were placed in (1) 11 almonds exposed to the sun most of the day, (2) 11 almonds exposed to the shade most of the day, and (3) two air locations shaded from sun, one in each tree. We compiled the average daily exposure time in intervals of 5°C from 15° to 60° for shade, sun, or air locations (appendix table 1).

Appendix table 1.--*Average daily exposure times, in minutes, for almonds in shaded or sunny locations and air in the tree canopy. Exposure is average daily exposure time in intervals of 5°C from 15° to 60°C. Temperatures were recorded for 27 days from 8 a.m. to 8 p.m. The temperatures were measured on the tree for 18 days (Aug. 8 to 26) and on the ground under a tree for 9 days (Aug. 27 to Sept. 4), 1977*

Location in orchard	Location where temperature was taken	Temperature interval								
		15°-19°C	20°-24°C	25°-29°C	30°-34°C	35°-39°C	40°-44°C	45°-49°C	50°-54°C	55°-60°C
		Minutes within each temperature interval								
Tree	air	2	55	155	421	90	0	0	0	0
Tree, fruit	shade	0	45	220	397	60	0	0	0	0
	sun	0	30	145	155	190	125	40	10	0
Ground	air	0	30	130	350	165	0	0	0	0
Ground, fruit	shade	0	30	170	305	165	20	0	0	0
	sun	0	5	60	70	85	135	185	130	10